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The Cell Death-promoting Gene DP5, Which Interacts with the BCL2 Family, Is Induced during Neuronal Apoptosis Following Exposure to Amyloid β Protein*

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DP5, which contains a BH3 domain, was cloned as a neuronal apoptosis-inducing gene. To confirm that DP5 interacts with members of the Bcl-2 family, 293T cells were transiently co-transfected with DP5 and Bcl-xl cDNA constructs, and immunoprecipitation was carried out. The 30-kDa Bcl-xl was co-immunoprecipitated with Myc-tagged DP5, suggesting that DP5 physically interacts with Bcl-xl in mammalian cells. Previously, we reported that DP5 is induced during neuronal apoptosis in cultured sympathetic neurons. Here, we analyzed DP5 gene expression and the specific interaction of DP5 with Bcl-xl during neuronal death induced by amyloid-β protein (A β). DP5 mRNA was induced 6 h after treatment with A β in cultured rat cortical neurons. The protein encoded by DP5 mRNA showed a specific interaction with Bcl-xl. Induction of DP5 gene expression was blocked by nifedipine, an inhibitor of L-type voltage-dependent calcium channels, and dantrolene, an inhibitor of calcium release from the endoplasmic reticulum. These results suggested that the induction of DP5 mRNA occurs downstream of the increase in cytosolic calcium concentration caused by A β. Moreover, DP5 specifically interacts with Bcl-xl during neuronal apoptosis following exposure to A β, and its binding could impair the survival-promoting activities of Bcl-xl. Thus, the induction of DP5 mRNA and the interaction of DP5 and Bcl-xl could play significant roles in neuronal degeneration following exposure to A β.

Apoptosis, or programmed cell death, plays an important role not only in neuronal development and differentiation of the central nervous system but also in the pathogenesis of a variety of neurodegenerative disorders such as Alzheimer's disease. However, the molecular events or cascades underlying neuronal death regulated by the genetic program still remain unclear. Elucidation of the molecular mechanisms underlying neuronal death could contribute to understanding of the pathophysiology of neurodegenerative disorders such as Alzheimer's disease.

Previously, we isolated a novel gene named DP5 that is induced during neuronal apoptosis using rat sympathetic neurons in culture deprived of NGF (1).[1] This gene has the following unique features: 1) the encoded protein has a BH3 domain, which is essential for interaction with Bcl-2 and Bcl-xl, and a transmembrane region at its C-terminal; 2) its expression shows marked induction with peak levels at 15 h after NGF withdrawal, concurrent with the time at which neurons are committed to die in the sympathetic culture model; and 3) overexpression of full-length DP5 in cultured neurons was sufficient to induce apoptosis. In the developing murine nervous system, DP5 mRNA was localized in several tissues such as the trigeminal and dorsal root ganglia and the anterior horn of the spinal cord, which are known to contain a number of apoptotic cells in the mouse embryo. These observations suggested that DP5 could be associated with the phenomena of neuronal death *in vivo* (2).

Recently, Inohara *et al.* (3) cloned the human gene Harakiri (Hrk), which physically interacts with Bcl-2 and Bcl-xl. The polypeptide encoded by Hrk has a BH3 domain and transmembrane region and is highly homologous with DP5 (72% identity), suggesting that DP5 and Hrk are homologues from different species. Nbk/Bik (4, 5) and Bid (6) were identified as proteins that contain only BH3, and both interact with members of the Bcl-2 family and have death-promoting activities. However, these proteins do not show any significant amino acid homology beyond the conserved BH3 domain.

Amyloid β protein (A β) damages and kills cultured neurons by a mechanism involving oxidative stress and disruption of cellular calcium homeostasis (7–12). Morphologically, this type of neuronal death shows hallmarks of apoptosis including cellular shrinkage, blebbing of the plasma membrane, nuclear condensation, and nucleosomal fragmentation (13–15). To gain insight into the neuronal responses to A β at the molecular level, analyses of the effects of A β treatment on neuronal gene expression *in vitro* have been carried out (16). Some gene expression patterns induced by A β treatment were markedly similar to those of sympathetic neurons deprived of NGF, suggesting that a genetic cascade is necessary for neuronal death following exposure to A β similarly to NGF-deprived neuronal death.

In the present study, we determined whether DP5 gene expression was closely associated with the process of neuronal apoptosis induced by A β in addition to sympathetic neuronal

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[1] The abbreviations used are: NGF, nerve growth factor; A β, amyloid-β protein; RT, reverse transcription; PCR, polymerase chain reaction; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside.

death as reported previously (1). Furthermore, the changes in binding between DP5 and the death repressor protein Bcl-xl were also examined in the culture model. We report here that DP5 mRNA expression was induced and the encoded protein interacted specifically with Bcl-xl during neuronal death following exposure to A β. Our *in vitro* results suggested that DP5 may have an important role in neuronal apoptosis induced by treatment with A β and in the neuronal loss associated with Alzheimer's disease.

EXPERIMENTAL PROCEDURES

Primary Culture and Treatment with A β—Primary cultures of neuronal cells were prepared from the cortex of fetal rats at 18 days of gestation. The dissected tissues were treated with papain (Sigma), 0.02% DL-cysteine-HCl, 0.02% bovine serum albumin, 0.5% glucose, and 0.1% DNase to dissociate the cells. Aliquots of 1×10^7 cells were plated in 10-cm dishes coated with poly-L-lysine and maintained at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. On the next day, the medium was changed to Dulbecco's modified Eagle's medium containing B27 supplement (Life Technologies, Inc.) in place of fetal calf serum, and culture was maintained for 5 days before A β stimulation. A β_{25-35}, A β_{1-40}, and A β_{40-1} (Bachem) stock solutions were prepared as 1-mM stocks in sterile distilled water. Cultured rat primary neurons were exposed to A β by the culture medium replacing with Dulbecco's modified Eagle's medium/B27 containing 20 or 40 μm A β. The numbers of living cells were counted based on morphological criteria and trypan blue staining at various time points after treatment with A β.

RT-PCR Analysis—Aliquots of 3 μg of total RNA purified from preplated cortical neuron cultures were reverse-transcribed using 300 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 60-μl reaction mixtures in the presence of 2.5 μM oligo(dT) primer and 20 μM dNTP mixture for 60 min at 37 °C. For PCR amplification, specific oligonucleotide primer pairs (0.5 μM each) were incubated with 1 μl of cDNA, 1 unit of *Taq* polymerase, 1 × *Taq* buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$), 10 μM dNTP mixture, and 10 μCi of [α-^{32}P]dCTP in a 20-μl reaction mixtures. The sequences of primers used in this study were as follows: DP5 sense primer, 5′-AGACCCAGCCCGGACCGAGCAA-3′, and DP5 antisense primer, 5′-ATAGCACTGAGGTGGCTATC-3′; neurofilament-M sense primer, 5′-TGGCTTAGATGTGAGCCCTG-3′, and neurofilament-M antisense primer, 5′-GACTATGGCATGTGAAGTGACC-3′; Bcl-2 sense primer, 5′-CTGGTGGACAACATCGCTCTG-3′, and Bcl-2 antisense primer, 5′-GGTCTGCTGACCTCACTTGTG-3′; Bcl-xl sense primer, 5′-AGGCTGGCGATGAGTTTGAA-3′, and Bcl-xl antisense primer, 5′-CGGCTCTCGGCTGCTGCATT-3′; Bax sense primer, 5′-TGGTTGCCCTTTTCTACTTTG-3′, and Bax antisense primer, 5′-GAAGTAGGAAAGGAGGCCATC-3′. Typical PCR parameters were 1 min at 94 °C, 1 min at 60 °C, and 30 s at 72 °C for 22–24 cycles followed by 72 °C for 5 min. Aliquots of 10 μl of each reaction mixture were electrophoresed through 5% polyacrylamide gels, and the dried gels were subjected to autoradiography. Control experiments were performed to determine the range of PCR cycles over which amplification efficiency remained constant. The identity of each PCR product was confirmed by subcloning the amplified cDNAs into the pGEM-T vector (Promega) and sequencing.

Interaction of DP5 and Bcl-2 Family—Aliquots of 1×10^7 primary neurons were plated on 10-cm dishes. Two dishes were used per immunoprecipitation. Cells were harvested and rinsed twice with PBS and then lysed in 1 ml of buffer containing 10 mM Tris-HCl (pH 7.8), 0.2% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 10 μg/ml aprotinin. Lysates were centrifuged at 13,000 rpm for 5 min to remove large cellular debris. For each immunoprecipitation experiment, 3 μg of antibody was used. Samples were incubated for 1 h at 4 °C on a rocker with the antibodies. Recombinant protein G agarose (Life Technologies, Inc.) was added to each sample followed by another 1-h incubation at 4 °C on a rocker. The beads were then washed five times in lysis buffer to reduce nonspecific binding. After the last wash, all buffer was removed, and reducing sample buffer was added to each reaction mixture. Samples were boiled and loaded onto a 5–20% gradient SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto Immobilon P membranes (Millipore). Blots were preblocked in PBS containing 5% nonfat milk, and washes were performed using PBS containing 0.1% Tween 20 (PBS-T). Primary antibodies were used at 0.2–0.5% (v/v), and detection was performed with 0.1% alkaline phosphatase-conjugated goat anti-rabbit IgG (Boehringer Mannheim) or goat anti-mouse IgG (Sigma) in PBS-T with 5% nonfat milk by the alkaline phosphatase method. Anti-Bcl-2 and Bcl-xl monoclonal antibodies were purchased from MBL (Japan). Clone 9E10 monoclonal antibody was used for detection of Myc epitope sequence. Anti-DP5 polyclonal antibodies were raised against the recombinant glutathione *S*-transferase-DP5 fusion protein (1) and peptide corresponding to amino acid residues 25–58 of DP5.

Plasmid Construction—The mammalian expression plasmids SFFV-human Bcl-2 and mouse Bcl-xl were provided by Prof. Nunez (University of Michigan Medical School). The Myc epitope sequence was attached to the rat DP5 cDNA to generate Myc-rat DP5 by PCR and

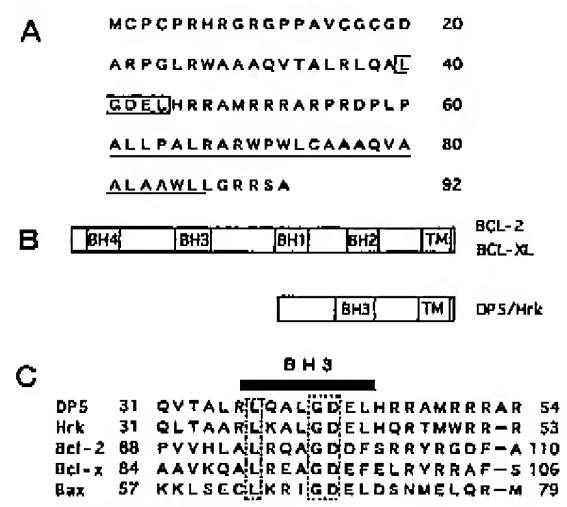

FIG. 1. **Structure of DP5 polypeptide.** *A*, amino acid sequence of DP5 polypeptide. DP5 consists of 92 amino acids. The conserved BH3 domain and putative transmembrane region are indicated by a *box* and *single underline*, respectively. *B*, schematic structures of DP5 and Bcl-2 family. *C*, comparison of the BH3 domains between DP5 and Bcl-2 family members. The amino acids surrounded by *dotted lines* are conserved in all members.

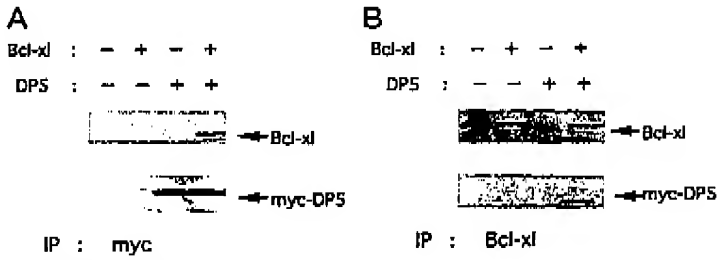

FIG. 2. **Interaction of DP5 and Bcl-xl in 293T cells.** 293T cells were transiently transfected with the indicated plasmids. The DP5 expression plasmid was tagged with the human Myc amino acid sequence at the N terminus of DP5. Transfection was performed with equal amounts of plasmid DNA using empty plasmid as a control. *A*, lysates were immunoprecipitated (*IP*) with anti-Myc antibody. Immunoprecipitates were immunoblotted with anti-Bcl-xl antibody (*upper panel*) and anti-Myc antibody (*lower panel*). *B*, immunoprecipitates with anti-Bcl-xl antibody were immunoblotted with anti-Bcl-xl antibody (*upper panel*) and anti-Myc antibody (*lower panel*).

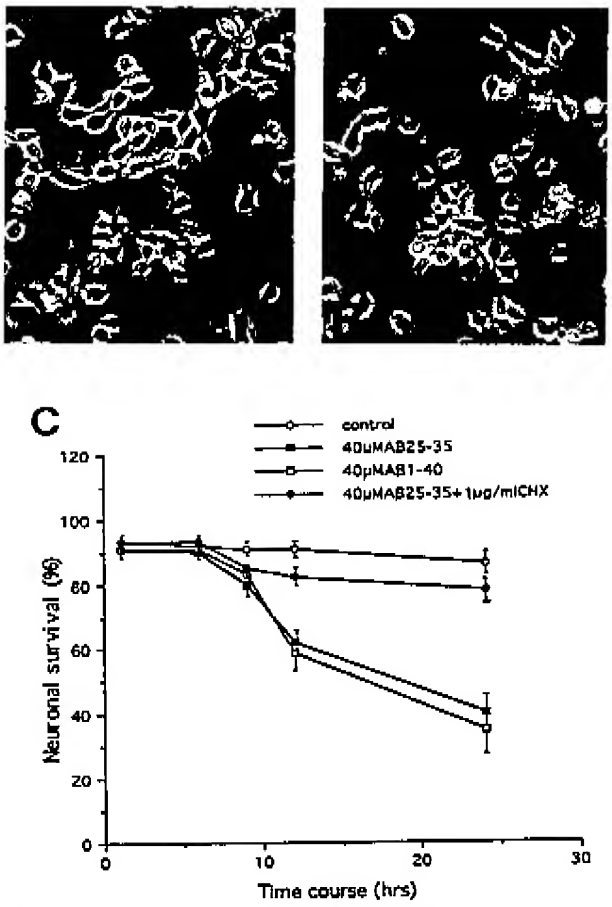

FIG. 3. **A β neurotoxicity in rat cortical neuronal culture.** Representative phase-contrast micrographs of cortical neurons. *A*, control culture. *B*, culture exposed to 40 μM A β_{25-35} for 24 h. A β-treated neurons underwent degeneration; degenerating neurons showed disruption of neurites, shrinkage and irregularly shaped cell bodies. *C*, time course of A β neurotoxicity. Culture neurons were exposed to 40 μM A β_{25-35} and 40 μM A β_{1-40} with or without cycloheximide (1 μg/ml). Changes in survival of neurons at the specified time points were determined. Results are presented as the mean percentages of surviving cells ± SD observed in five independent experiments.

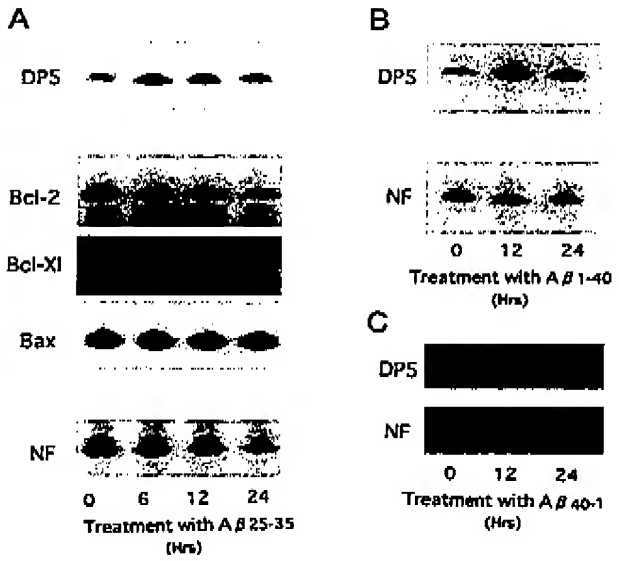

FIG. 4. **RT-PCR analysis of DP5 mRNA expression after treatment with A β.** Cortical neurons were exposed to 40 μM A β_{25-35} (*A*), A β_{1-40} (*B*), and A β_{40-1} (*C*) for the indicated intervals. Total RNA was isolated at each time point and then reverse-transcribed followed by PCR analysis for DP5 and Bcl-2 family members as described under "Experimental Procedures." Neurofilament-M (*NF*) was used as an internal marker.

cloned into pCDNA3 or pIND (Invitrogen).

Human embryonic kidney 293T cells were used for transient transfection. 10-cm culture dishes containing 5×10^6 cells were transfected with 5 μg of plasmid DNA by lipofection (LipofectAMINE, Life Technologies, Inc.). The levels of expression of each protein were determined in total lysates by Western blotting. For inducible expression, transfection of plasmids and induction of expression were performed according to the supplier's recommendations. Briefly, Myc-DP5 cloned into pIND was cotransfected with pVgRXR into 293T cells. On the next day, cells were treated with 3 μM muristerone A (Invitrogen) to induce intracellular expression from pIND.

X-Gal Staining—For X-gal staining of cells expressing β-galactosidase, cells were fixed in 1% glutaraldehyde for 3 min and stained with X-gal solution (100 mM sodium phosphate buffer (pH 7.2), 10 mM KCl, 1 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, and 0.1% X-gal) at 37 °C.

RESULTS

Structure of DP5 Polypeptide and Its Interaction with Members of the Bcl-2 Family—DP5 has a BH3 domain, which was shown to be essential for the interaction with Bcl-2 and Bcl-xl proteins (Fig. 1). Recently, Hrk, which was considered to be a human DP5 homologue, was shown to interact with Bcl-2 and Bcl-xl by *in vitro* transfection analysis (3). To confirm that rat DP5 interacts with members of the Bcl-2 family, 293T cells were transiently co-transfected with expression plasmids producing Myc-tagged DP5 and Bcl-xl. Immunoprecipitates were prepared using anti-Myc monoclonal antibody and subjected to immunoblotting with anti-Bcl-xl antibody. Western blotting with anti-Bcl-xl antibody revealed that 30-kDa Bcl-xl was co-immunoprecipitated with Myc-DP5 (Fig. 2*A*). As the reverse experiment, we performed immunoprecipitation using anti-Bcl-xl antibody, followed by blotting with anti-Myc antibody. The 10-kDa Myc-DP5 was co-immunoprecipitated with Bcl-xl (Fig. 2*B*). To examine the interaction of Bcl-2 and DP5, we performed immunoprecipitation experiments similar to those used to assess the DP5-Bcl-xl interaction. Our results confirmed that DP5 specifically interacted with Bcl-2 (data not shown). However, we detected only a faint band of Bcl-2, which interacted with Myc-DP5 in contrast with the results of immunoprecipitation with anti-Bcl-xl antibody. Accordingly, we examined only the interaction of DP5 with Bcl-xl in the next set of experiments.

Neuronal Death by A β Toxicity—To determine the neuronal toxicity of A β, cortical neurons were exposed to A β_{25-35} or A β_{1-40} at a concentration of 40 μM. Viability was quantified by trypan blue staining and morphological criteria at various time points. Neurons began to degenerate asynchronously, exhibiting shrinkage and irregularly shaped cell bodies with dystrophic neurites from about 12 h after A β exposure (Fig. 3). At 24 h, cultures treated with both A β_{25-35} and A β_{1-40} showed 40% cell survival compared with controls. Neuronal death was prevented by treatment with cycloheximide, a protein synthesis inhibitor, at the same time as addition of A β. These results suggested that A β-induced cell death is dependent on macromolecular synthesis and is controlled by a genetic program. Our observations were consistent with those of previous studies (13, 16, 17).

Expression of DP5 mRNA during Neuronal Death—To determine the temporal changes in levels of DP5 mRNA during

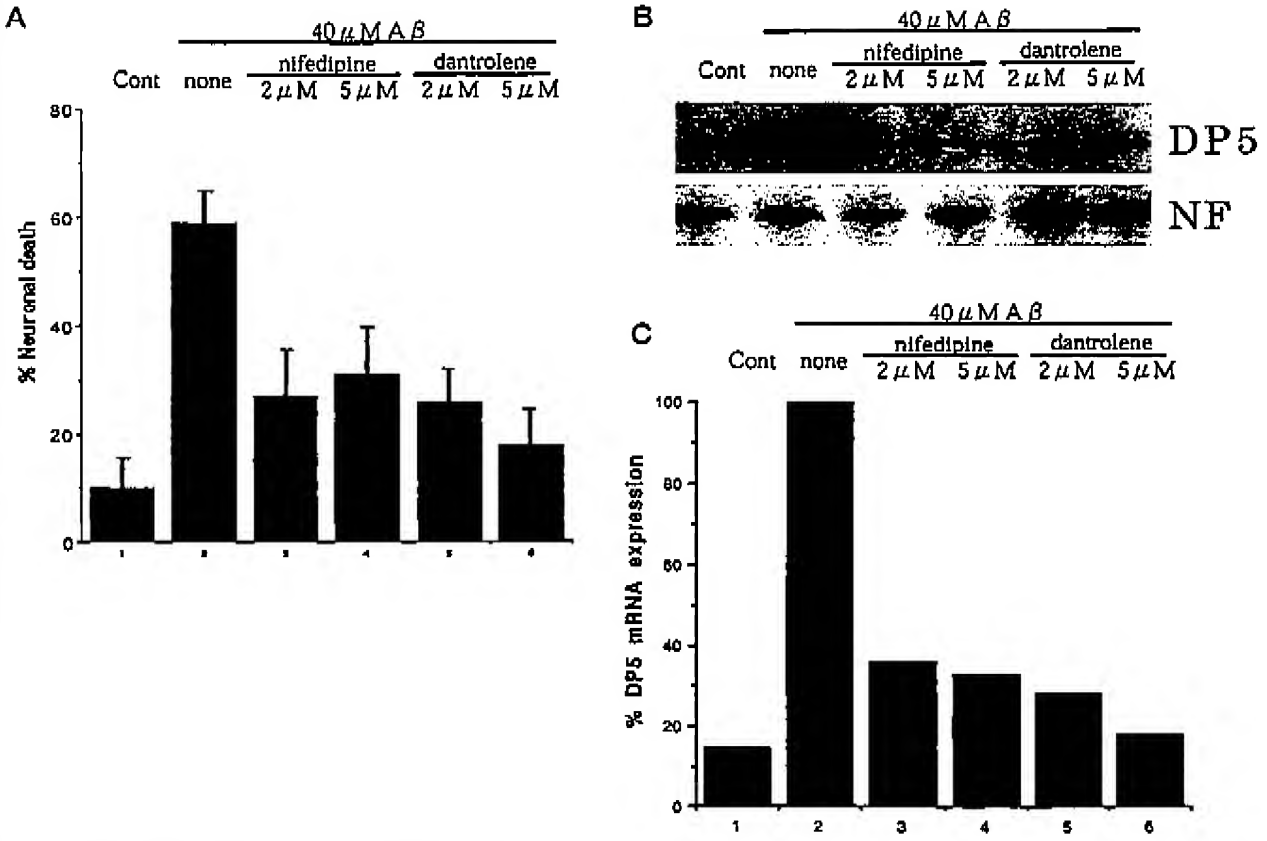

FIG. 5. **DP5 mRNA expression on treatment of cortical neurons with agents that prevent neuronal death induced by A β_{25-35}.** *A*, after cortical neurons were treated with 40 μM A β_{25-35} for 12 h in the presence or absence of 2 or 5 μM nifedipine or 2 or 5 μM dantrolene, the numbers of dying cells were counted. The data (means ± S.D.) shown are percentages of dead cells. Data were collected from at least five independent experiments. *B*, RT-PCR analysis of changes in DP5 expression 6 h after exposure to 40 μM A β_{25-35} in the presence of cell death blockers. Neurofilament-M was used as an internal control. *C*, quantification of changes of DP5 expression suppressed by cell death blockers. Changes in the levels of DP5 were quantified by Scanning Imager (Molecular Dynamics) analysis of polyacrylamide gels such as that shown in *B*. *Cont*, control.

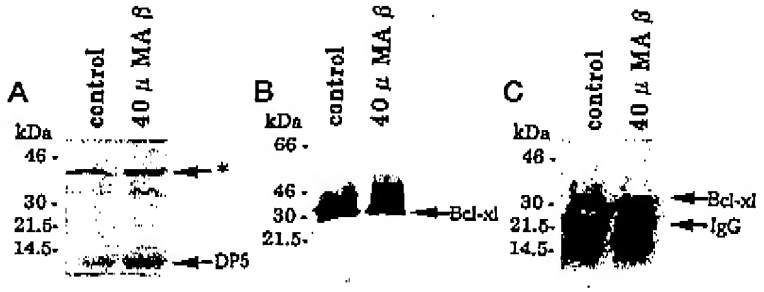

FIG. 6. **Interaction of DP5 and Bcl-xl during neuronal death induced by A β_{25-35}.** Primary cultures were maintained in the presence or absence of 40 μM A β for 6 h before harvesting. Following immunoprecipitation with anti-recombinant DP5 antibody, samples were subjected to immunoblotting analysis using anti-Bcl-xl antibody. Although control lysates did not show a 30-kDa Bcl-xl band, lysates from neurons treated with A β for 6 h showed a single Bcl-xl band (*C*). Western blotting showed that DP5 protein was induced in cortical neurons treated with A β (*A*), and the amounts of Bcl-xl protein in both cell lysates were equivalent (*B*). The *asterisk* indicates a nonspecific band.

neuronal death induced by A β, we performed reverse transcription of mRNAs isolated from cortical neuronal cultures before and at various time points after A β treatment and analyzed the proportions of DP5 cDNAs obtained using RT-PCR (Fig. 4). We also analyzed the expression patterns of members of the Bcl-2 family including Bcl-2, Bcl-xl, and Bax. DP5 expression level was relatively low before A β stimulation. This signal increased at 6 h after addition of 40 μM A β_{25-35}, and the level was maintained at least until 12 h, showing a subsequent reduction after this time point that appeared to be consistent with neuronal loss (Fig. 4*A*). Stimulation with A β_{25-35} and A β_{1-40} showed almost equivalent induction of DP5 mRNA (Fig. 4*B*). Moreover, when cortical neurons were exposed to A β_{25-35} or A β_{1-40} at a concentration of 20 μM, the pattern of expression of DP5 mRNA was the same as that at 40 μM (data not shown). On the other hand, the levels of Bcl-2

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family mRNA expression did not change or were slightly diminished during the course of cell death, and DP5 mRNA expression was not induced by treatment with 40 μM A β_{40-1} (Fig. 4*C*). These results suggested that within the Bcl-2 family, DP5 mRNA was selectively induced during A β-induced neuronal death.

Previous studies showed that disruption of cellular calcium homeostasis occurs in neuronal apoptosis induced by A β (7, 11, 17–19) or deprivation of NGF (20, 21). Indeed, nifedipine, a blocker of L-type voltage-dependent calcium channels (22), and dantrolene, an inhibitor of calcium release from ER stores (23), prevent neuronal death induced by A β. We next examined the changes in DP5 mRNA expression following treatment of cortical neurons with nifedipine or dantrolene. To test the abilities of these two agents to promote survival, cortical neurons treated with 40 μM A β_{25-35} were cultured for 24 h with or without these agents, and the numbers of surviving neurons were counted. Without additives, approximately 60% of neurons died following exposure to A β, whereas neuronal death was prevented in the presence of nifedipine or dantrolene (Fig. 5*A*). After cortical neurons were treated with 40 μM A β for 6 h in the presence or absence of these agents, total RNA was extracted and analyzed for changes in DP5 gene expression by RT-PCR. Levels of products amplified by DP5-specific primers were markedly decreased in all cases treated with these two agents (Fig. 5*B*). Quantification of the DP5 signals showed that the expression levels of DP5 in cortical neurons treated with calcium blockers were decreased to approximately the same levels as in nontreated controls (Fig. 5*C*).

Interaction of DP5 and Bcl-2 Families during Neuronal Death Induced by A β—To determine whether DP5 interacts with members of the Bcl-2 family during neuronal apoptosis following exposure to A β, we performed immunoprecipitation followed by immunoblotting analysis of DP5 and Bcl-xl after treatment with A β (Fig. 6). The expression of DP5 protein was increased in cortical neurons 6 h after exposure to A β_{25-35}, consistent with the DP5 mRNA expression pattern (Fig. 6*A*). DP5 did not bind with Bcl-xl before A β stimulation. At 6 h after treatment with A β, a 30-kDa band of Bcl-xl was detected in immunoprecipitates by anti-DP5 antibody (Fig. 6*C*). The expression levels of Bcl-xl proteins were equivalent in A β-stimulated and nonstimulated cultured neurons (Fig. 6*B*). These results suggested that DP5 specifically interacted with Bcl-xl during neuronal death induced by A β.

Mechanisms of Cell Death Induced by the Expression of DP5—Stimulation with A β resulted in an increase in level of DP5 mRNA within 6 h. A specific interaction between DP5 and Bcl-xl also occurred in cell death induced by A β. Our previous study indicated that overexpression of DP5 in cultured neurons was sufficient to induce cell death. Taken together, these observations suggested that increased expression of DP5 and its interaction with Bcl-xl play a significant role in the process of neuronal death. To examine the events involved in DP5-induced cell death after interaction with Bcl-xl, we analyzed the characteristics of DP5 expression-dependent cell death using ecdysone-inducible expression systems (24). 293T cells were transiently transfected with a pIND-derived plasmid expressing Myc-DP5 and pVgRXR plasmid. After culture for 24 h, cells were incubated in the presence of 3 μM muristerone A, an ecdysone analogue, for various periods. On addition of muristerone A, 293T cells rapidly began to undergo apoptosis within 6 h and showed <40% viability at 24 h (Fig. 7). The cell death was inhibited by the expression of Bcl-xl. Western blotting analysis revealed induction of DP5 by 1 h, and the level of expression was maintained up to 24 h after treatment with muristerone A.

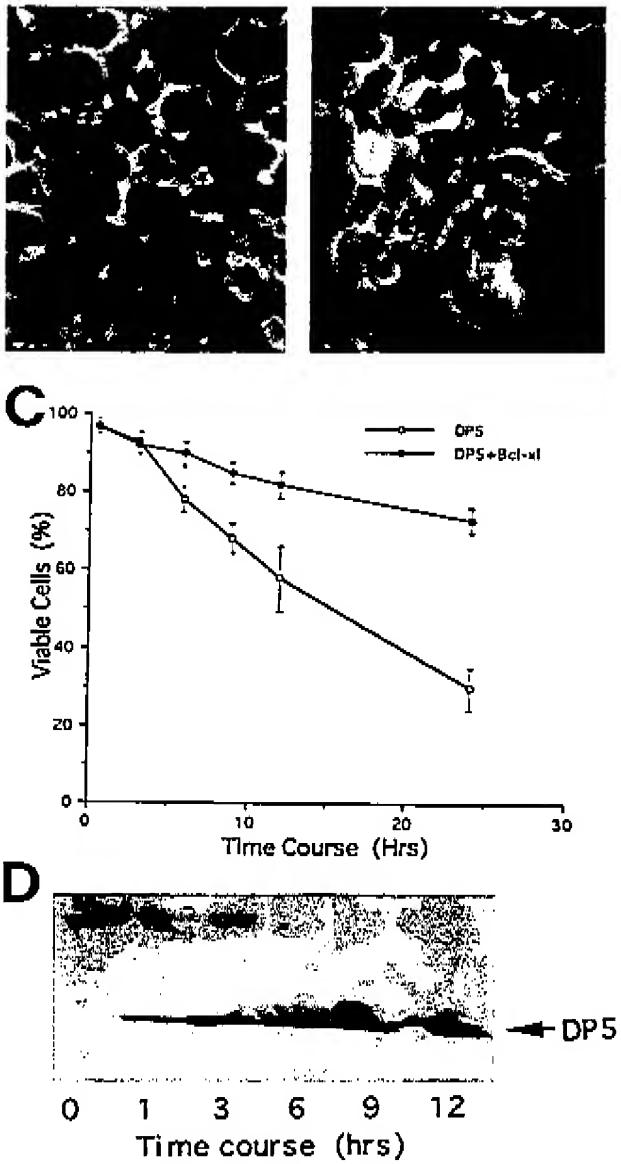

FIG. 7. **DP5 induces apoptosis in 293T cells.** *A* and *B*, phase-contrast photographs of 293T cells after induction of DP5 in response to muristerone treatment (X-gal staining). X-gal-stained DP5-expressing 293T cells showed shrinkage and blebbing (*B*), and these cells increased in number with time after induction, whereas the Bcl-xl expression plasmid pSFFV/Bcl-xl that was co-transfected with the DP5-inducible plasmid showed intact morphology (*A*). *C*, time course of changes in number of living cells. The percentages of blue intact cells are shown. The data are the means ± SD from 10 fields counted in three independent transfection experiments. *D*, Protein immunoblotting analysis of DP5 expression after induction by muristerone. Lysates of cells at various time points after muristerone-induction were subjected to Western blotting analysis using anti-DP5 antibody.

Cell death was significantly attenuated in the Ca^{2+}-depleted state by exposure to 1 mM EGTA (Fig. 8*B*), which provided preparations with low cytosolic and low sequestered Ca^{2+} (22). Dantrolene also prevented cell death. In contrast, depolarization by high potassium (35 mM) or nifedipine did not prevent cell death induced by expression of DP5, suggesting that the cell death involves calcium release from ER and the resultant disruption of calcium homeostasis causes cell death.

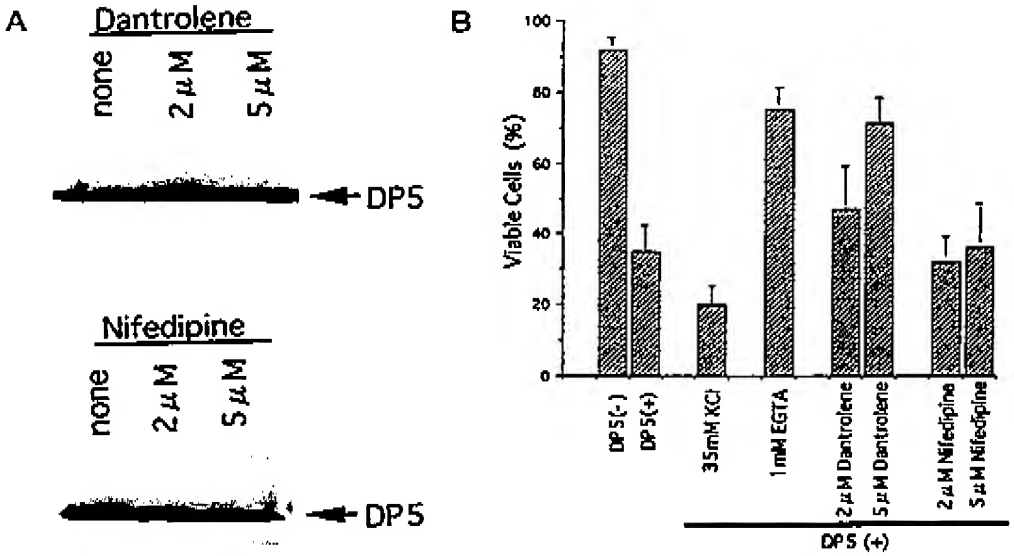

FIG. 8. **The effects of various agents on DP5-induced cell death.** *A*, the expression of DP5 protein 6 h after addition of muristerone with dantrolene or nifedipine. The amounts of DP5 protein in each cell lysate were equivalent. *B*, cell death prevented by various agents. Living cells were counted 24 h after addition of muristerone in the presence of 35 mM KCl, 1 mM EGTA, 2 or 5 μM dantrolene, or 2 or 5 μM nifedipine (means ± S.D.).

DISCUSSION

Previous studies showed that A β induces apoptosis in neurons in primary culture (13, 16, 17). The mechanisms of A β neurotoxicity involve membrane lipid peroxidation and impairment of intracellular calcium homeostasis (7–12). However, the detailed mechanisms are still unclear. We found that the messenger RNA of DP5, which has been cloned as a gene induced in programmed cell death of sympathetic neurons deprived of NGF (1), was selectively elevated after A β stimulation. Recently, gene expression patterns during neuronal death induced by treatment with A β were reported to be markedly similar to those observed in the models of sympathetic neurons deprived of NGF, *i.e.* induction of *c-jun* begins in the early stage followed by *c-fos*, *fos*B at the time of commitment to cell death in both culture models (16, 25). The temporal patterns of expression of several genes including DP5 during neuronal death after A β treatment indicated that A β stimulus activates a cellular genetic program for cell death similarly to NGF deprivation.

Calcium influx contributes to A β-induced neuronal degeneration because removal of extracellular calcium (26) and calcium channel blockers (18, 19) protect neurons against A β toxicity. So we examined whether the DP5 gene induction after exposure to A β was changed following treatment with these agents. Cell death was prevented by treatment with nifedipine and dantrolene, which are blockers of L-type voltage-dependent calcium channels and of calcium release from the ER, respectively. In these cases, the expression of DP5 mRNA was significantly suppressed, suggesting that induction of DP5 mRNA occurs downstream of the increase in cytosolic calcium concentration caused by A β. We considered that A β stimulus caused the influx of extracellular calcium, calcium release from the ER, and accumulation of reactive oxygen species, followed by activation of the apoptosis cascade involving induction of cell death-promoting genes such as DP5.

The protein encoded by DP5 mRNA contains a BH3 domain that is critical for interaction with members of the Bcl-2 family and regulation of apoptosis. *Harakiri* (Hrk), which was reported to bind to members of the Bcl-2 family (3), is considered to be a human homologue of DP5 because it shows 72% overall amino acid sequence identity and the sequence of the BH3 region is completely conserved. Hrk physically interacts with Bcl-2 and Bcl-xl at the BH3 region. We confirmed that DP5 also possessed the ability to interact with Bcl-2 family members. Nbk/Bik, Bid, and Hrk are known as proteins that contain only BH3 and were identified recently as interacting partners with Bcl-2 family members (3–6). Overexpression of these proteins including DP5 is sufficient for induction of apoptosis in several types of cells. The mechanisms by which these molecules induce apoptosis are not well known. There are two possible mechanisms as follows: proteins that contain only BH3 may be death effector molecules, *i.e.* Bcl-2 or the other Bcl-2 family members may be dominant negative regulators; alternatively, these molecules may promote cell death by inhibiting the death-suppressing activities of the Bcl-2 family. In the present study, the level of expression of DP5 in neurons was low under normal conditions. Death signals such as A β stimulus caused the accumulation of DP5 mRNA, but levels of expression of Bcl-2 family members were not changed or were diminished. Furthermore, the protein encoded by DP5 mRNA interacted with Bcl-xl during cell death. These observations lead us to hypothesize the scenario as follows: A β stimulus causes the selective accumulation of DP5 mRNA by increasing intracellular calcium concentration involved in activation of the cell death cascade followed by interaction of Bcl-2 family members and DP5 protein via the BH3 region. Binding to the BH3 domain could impair the survival-promoting activities of the Bcl-2 family (27, 28). However, we cannot exclude the possibility that DP5 could be an effector molecule because overexpression of Bcl-xl inhibited the killing activity of DP5.

To examine the molecular events involved in DP5 expression-dependent cell death, we established an ecdysone-inducible expression system for DP5. 293T cells expressing DP5 rapidly underwent apoptosis in this system, and this cell death was blocked by perturbation of intracellular calcium concentration. Dantrolene, which is an inhibitor of calcium release from the ER and which is known to protect neurons against A β toxicity (29), and EGTA prevented cell death induced by DP5. These agents provide the situation of low intracellular concentrations of calcium. In contrast, nifedipine, a blocker of L-type voltage-

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dependent calcium channels, had no effect. These results indicated that DP5-induced cell death involves calcium release from ER, but not calcium influx through plasma membrane channels. Previous studies have shown that ER calcium regulation contributes to apoptosis of neuronal (29) and nonneuronal cells (30, 31). Moreover, Bcl-2 protects lymphoma cells against apoptosis induced by thapsigargin, an inhibitor of ER calcium-ATPase, and suppresses release of calcium from the ER (32, 33). Collectively, overexpression of DP5 could affect intra- and extra-ER calcium homeostasis, and the resultant increase in cytosolic calcium concentration could lead to apoptosis. The localization of DP5 and its human homologue, Hrk, to the membranes of intracellular organelles (3) supports the above hypothesis. However, it is unclear whether DP5 can itself generate channels on the membranes similarly to Bax (34) or activate the IP3 pathway and how DP5 accelerates the calcium release from ER after increase of cytosolic calcium concentration in neuronal death induced by A β.

In conclusion, the present study strongly suggested that DP5 plays a significant role in neuronal apoptosis followed by exposure to A β. We are currently engaged in generation of DP5 knockout mice to examine the resistance to A β toxicity using neurons derived from the mouse brain. These experiments will help to elucidate the molecular mechanisms underlying neurodegeneration induced by A β and may allow for the development of therapeutic strategies for Alzheimer's disease.

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